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SHORT COMMUNICATIONS

Effects of phenothiazines on the development of antibody-producing cells *in vitro**

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In the last decade phenothiazines have been used widely in the chemotherapy of psychoneurotic disorders.[1,2] Occasionally, their use is accompanied by and associated with the clinical appearance of bone marrow depression and aplastic anemia.[3,4] Henson *et al.*[5] have demonstrated that propiomazine, a phenothiazine derivative, is a potent agent for the experimental production of the "runting syndrome" in newborn rabbits indicating that the development of immune competence is suppressed. For these reasons, we decided to study the development of immunity *in vitro*, using the spleen cell culture technique of Mishell and Dutton.[6] To explore the mechanism of action of phenothiazines at the cellular level, some experiments were carried out using a modification of the cell separation technique of Mosier,[7] in which the spleen cells are divided into two populations on the basis of their ability to adhere to plastic. Also, to assess the effect of chlorpromazine on the antigen(s), sheep erythrocytes were treated with the drug using several modifications of the method of Seeman and Weinstein.[8]

Hybrid male mice of the LAF_1 (C57L × A/He) strain purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine, between the ages of 8 and 16 weeks, were used for these experiments. Fetal calf serum was obtained from Armour Pharmaceuticals, Kankakee, Ill.; guinea pig complement and sheep red blood cells were obtained from the Colorado Serum Company, Denver, Colo. Concentrates of vitamins, essential amino acids, non-essential amino acids, glutamine, sodium pyruvate and guinea pig complement were obtained from Gibco, Grand Island, N.Y.

Cell culture conditions for the induction of hemolysin plaque-forming cells were described by Mishell and Dutton.[6] Procedures for the determination of hemolysin plaque-forming cells (PFC)† by the assay of Jerne *et al.*[9] are given in an earlier study. As a routine procedure, $1{\cdot}5$–$2{\cdot}0 \times 10^7$ dispersed spleen cells were planted, and drugs were added at varying time intervals. Cells were harvested and assayed for PFC on the fourth day after planting.

In order to determine cytotoxic effects of the drugs, cell viability was determined by the nigrosin method[10] and the number of recovered spleen cells at the time of assay were counted in a Spencer hemocytometer.

Spleen cells were separated into adherent (M) and nonadherent (L) cells by a modification of the method of Mosier.[7] Chlorpromazine was added at appropriate concentrations to one or the other cell fraction and to both cell fractions in a given experiment. Incubation of the separated cell fractions with chlorpromazine was conducted at 37° in BSS and 10% fetal calf serum. After 60 min of incubation, L cells contained in test tubes were centrifuged at 4° at 900 rpm for 10 min in an International model PR2 centrifuge and resuspended in BSS. After removing the supernatant solution by suction, cells were washed two times with 2·0 ml of BSS containing 10% fetal calf serum. For M cells adhering to dishes, the supernatant fluid containing drug was removed by suction, washed two times with 1·0 ml of BSS, swirled, and removed again by suction. The two cell fractions were then added together in culture media and incubated in the usual way.

4×10^8 sheep erythrocytes (SRBC)/ml were incubated under several conditions of temperature and time in BSS with chlorpromazine at varying concentrations. At the end of incubation SRBC were washed three times and resuspended in BSS in a volume equivalent to the initial SRBC concentration. To replicate control cultures, approximately 4×10^7 SRBC were added dropwise. Similarly, CPZ-treated SRBC were also added in a volume equal to that of control. The cell cultures were then incubated in the usual way.

All drugs used for the experiments were dissolved in BSS and added by micropipette to give the final concentration specified in the text. Chlorpromazine hydrochloride was obtained from Smith, Kline & French Laboratories, promazine hydrochloride from Wyeth Laboratories and tri-flupromazine hydrochloride from Squibb Laboratories. All drugs were stored in the dark at 4°.

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† Abbreviations used: PFC, plaque-forming cell; CPZ, chlorpromazine; L and M, lymphocyte-rich and macrophage-rich; BSS, Hank's Balanced Saline Solution; SRBC, sheep red blood cells.

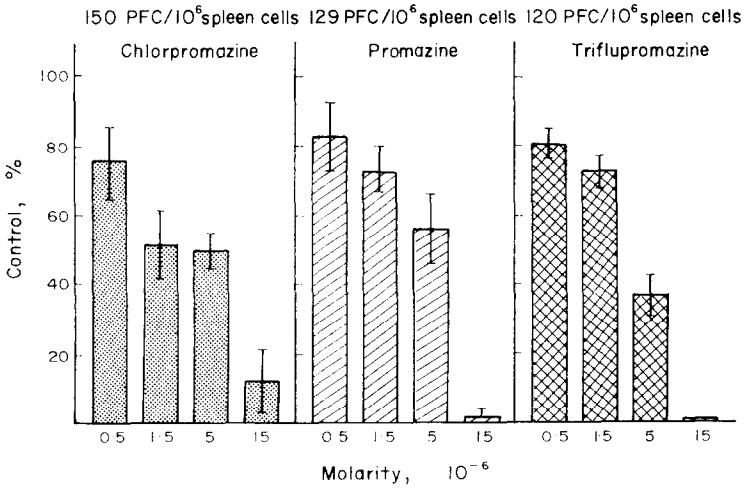

FIG. 1. Effect of different concentrations of chlorpromazine, promazine and triflupromazine on the production of PFC *in vitro* when drugs were added on day 0 of cultivation.

Addition of the three phenothiazine derivatives to spleen cultures on the day of explantation was inhibitory to the development of PFC at low molar concentrations of the drugs. As shown in Fig. 1, chlorpromazine, promazine and triflupromazine were added to spleen cell cultures at 0·5, 1·5, 5·0 and 15×10^{-6} M on the day of explantation. For the most part, the three phenothiazines were equally effective in reducing the production of PFC from 0·5 to $15{\cdot}0 \times 10^{-6}$ M. However, triflupromazine at 5×10^{-6} M was significantly more inhibitory than chlorpromazine and promazine ($P < 0{\cdot}05$). This compares favorably with the suppression of development seen in other model developmental systems (e.g. axolotl[11] and chick embryos[12]).

TABLE 1. DAILY ADDITION OF 5×10^{-6} M CPZ ON PFC PRODUCTION AND CELL VIABILITY

	PFC production Control*	Viability†
Day 0	51 ± 5·5	72 ± 6·5
Day 1	76 ± 6·0	84 ± 2·5
Day 2	84 ± 15·0	87 ± 3·5
Day 3	88 ± 3·5	86 ± 2·0
No addition	100	88 ± 3·5

* Values represent the mean of three experiments ± standard error of the mean.

† Values are expressed as per cent viability and represent the mean of three experiments ± standard error of the mean.

In order to determine whether the inhibition of PFC production was related to the time of drug addition, chlorpromazine was added at 5×10^{-6} M on days 0, 1, 2 and 3 (Table 1); day 0 addition of the drug reduced PFC production to 51 per cent of control values indicating that the sensitive period for the inhibitory effects of chlorpromazine was day 0. This sensitivity to drug effect at a discrete period of time is a common attribute of most developmental systems *in vivo*.[11] Studies performed previously in the spleen cell system *in vitro* showed a similar sensitive period for antibiotics and antitumor agents.[13] However, addition of the drug on day 2 or 3 reduced PFC production by only 12–16 per cent. To assess whether the greater degree of inhibition observed with drug addition on day 0 was related to cytotoxicity, viability of the recovered spleen cells on day 4 of incubation was determined by the

nigrosin method (Table 1). As compared to the control, there was a 16 per cent reduction in cell viability for the drug applied on day 0 at 5×10^{-6} M ($P < 0{\cdot}05$); no significant differences in viability was observed with drug addition on day 1, 2 or 3 when compared with controls.

Both adherent (M) and non-adherent (L) spleen cells have been shown by Mosier[7] to be required for PFC production; it was of interest to determine which of the two functionally different cell types was affected by chlorpromazine pretreatment. Preincubation of either cell types (L or M) and reconstitution with the untreated partner produced stimulation of PFC production in all combinations. On the basis of 2–4 experiments, L plus chlorpromazine-pretreated M and chlorpromazine-pretreated L + M cells stimulated PFC production to levels which were 50 per cent above control cultures; with drug pretreatment of both fractions a lesser stimulation was observed (30 per cent increase over controls).

Observations by Seeman and Weinstein[8] on the lytic and stabilizing effects of chlorpromazine on the human erythrocyte membrane may enable us to gain some insight in regard to the data we have obtained. They showed a concentration-dependent biphasic effect of the drug, viz. lysis and stabilization of the human erythrocyte. They also found that the drug adsorbs to the erythrocyte membrane in saline solution without prior tanning,[8] and that at low concentrations the extent of drug adsorption was temperature-dependent.[14] We studied the effects of prior incubation of sheep red cells with 10^{-5} and 10^{-6} M chlorpromazine; after pretreatment for 1- and 15-hr incubation with the drug, followed by several washes with BSS, the treated SRBC were used as antigen for the culture of PFC *in vitro*. The results (not shown) indicated that 1-hr preincubation of SRBC did not significantly alter the production of PFC. However, a 15-hr preincubation at 37° resulted in PFC production which was 50 per cent above control values at 10^{-5} and 10^{-6} M chlorpromazine.

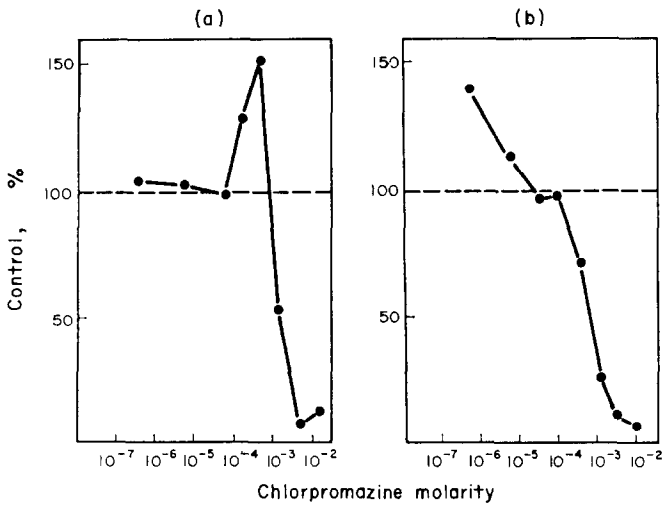

FIG. 2. (a) Effects of CPZ-treated SRBC on PFC production. Chlorpromazine at concentrations listed in the graph above was incubated with 4×10^8 SRBC for 10 min at room temperature. The unabsorbed drug was then removed by centrifugation and washed three times. Approximately 4×10^7 SRBC was then added to quadruplicate control spleen cultures, and equivalent volume suspensions of treated SRBC were added to the experimental group on duplicate cultures. (b) Effects of CPZ-treated SRBC kept at 4° for 3 days prior to use on PFC production. Washed samples of SRBC treated at room temperature with CPZ were left in the refrigerator for 3 days until used; they were then added to replicate cultures. (See text of Fig. 2a for procedural details).

Since the effect above is dependent on drug concentration, time and temperature of SRBC incubation, we studied the effects of preincubation at two temperatures and two intervals of incubation. The results of these studies are shown in Fig. 2. With a 10-min preincubation of SRBC at room temperature (Fig. 2a) over a wide range of drug concentrations a triphasic curve was observed in PFC production; i.e. an inhibitory phase followed by a stimulatory phase which, in turn, was followed by a return to control values. During the preincubation period, lysis of the SRBC occurred at drug concentrations ranging from 1×10^{-2} to 1×10^{-3} M; lysis was concomitant with inhibition of PFC production. At

5×10^{-4} M concentration, chlorpromazine did not produce any observable lysis, and at this preincubation concentration maximal PFC production was observed. With lower preincubation concentrations of chlorpromazine, less enhancement of PFC was seen. At 5×10^{-6} and 5×10^{-7} M, the lowest drug concentrations used, PFC production was equivalent to control values. However, when treated and washed SRBC were allowed to remain at 4° for 3 days, additional lysis of SRBC occurred at 1×10^{-4} M. As shown in Fig. 2b, no enhancement of PFC production occurred at this concentration. Inhibition of PFC was seen at 5×10^{-4} M and higher concentrations of the drug. On the other hand, enhancement of PFC occurred at 5×10^{-6} and 5×10^{-7} M. Similarly, Braun *et al.*[15,16] found that concomitant administration of chlorpromazine with sheep red cells produced enhancement of PFC production *in vivo*.

These findings point out that alterations of the membrane structure of either the erythrocyte antigen or the developmental cell type of the antibody-producing system are determinants in the differentiation of the antibody-producing cell.

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Purification and substrate specificity of uterine catecholamine oxidase

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PREVIOUS reports from this laboratory have described progress in the isolation, purification and characterization of an epinephrine-oxidizing enzyme which is present in a number of mammalian smooth muscles.[1–5] It was also observed that a product of the oxidation reaction inhibited uterine and cardiac actomyosin adenosine triphosphatase (ATPase) activity. The ATPase inhibitor was identified as a zwitterion, indole isomer of adrenochrome.[1,2] This enzyme system was shown to catalyze both the oxidation of epinephrine to adrenochrome and the isomerization of adrenochrome to a zwitterion isomer.[2,3]